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J. Sealey, A.J. Ragauskas, and T.J. Elder

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Investigations into Laccase-Mediator Delignification of Kraft Pulps

By J. Sealey[1], A.J. Ragauskas[1], and T.J. Elder[2]

[1]Institute of Paper Science and Technology, Atlanta, Georgia, U.S.A.

[2]School of Forestry, Auburn University, Auburn, Alabama, U.S.A.

Summary

The structure activity effects of N-hydroxy benzotriazole and phthalimide derivatives as mediators for laccase were studied. Using a softwood kraft pulp it was shown that the N-hydroxy unit must remain structurally intact for efficient laccase mediator delignification to occur. It was also found that the N-hydroxybenzotriazole structure was very sensitive to substituent effects with respect to laccase-mediator delignification. Computational results from PM3 indicate that the bond dissociation energy, and electronic factors of the radical may contribute to the efficiency of the mediator for LMS delignification.

Keywords Laccase, Mediators, Biobleaching, Delignification

Running Title: Laccase-Mediator Delignification Studies

Introduction

Bleaching of kraft pulps has changed substantially over the last decade in response to environmental and societal issues. Despite these advances, it is anticipated that further changes in pulp bleaching will occur as new technologies are developed that offer the opportunity for improved environmental and cost performance properties. The use of biological treatments for bleaching kraft pulps provide an alternative means of bleaching kraft pulps that could potentially yield distinct operating benefits. Indeed, xylanase (Santiago et al., 1995) and cellulase (Eriksson and Adolphson, 1997) are two bio-based commercial products that are currently employed for the production of paper.

Over the past decade the use of oxidative enzymes as replacements for chemical bleaching agents has shown significant advancement (Paice et al., 1994). Of the many oxidative enzymes under study, laccase has recently drawn the attention of several investigators. Although the redox chemistry of laccase has been extensively studied in wood formation (Dean and Eriksson, 1994) and degradation (Eriksson et al., 1990), it has been only over the last decade that a potential role for this enzyme has been proposed for bleaching kraft pulps. Initially, the use of laccase as a bleaching agent was not actively pursued since the enzyme does not readily degrade lignin because its size does not permit diffusion through pulp fibers (Jurasek, 1995). Studies by Bourbonnais and Paice (1992)

demonstrated that this size limitation could be circumvented if the bleaching reaction was performed in the presence of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). ABTS is believed to act as a type of cofactor in which laccase oxidizes ABTS and the oxidized ABTS diffuses into the fiber wall and oxidizes lignin. The reduced ATBS eventually diffuses from the fiber, gets re-oxidized by laccase and then further oxidizes lignin in the pulp. The chemical factors contributing to the laccase/ABTS delignification effect have been extensively examined. Recently, several other mediator structures (Amann, 1997; Call and Mucke, 1996) have been reported to enhance laccase delignification of kraft pulps. Although the relative efficiency of many of these mediators remains to be established, in our hands (see Figure 1) we have observed that N-hydroxybenzotriazole is a better mediator for laccase delignification of kraft pulps than ABTS (Sealey and Ragauskas, 1998).

Recently, the mechanism by which laccase/N-hydroxybenzotriazole (LMS system) delignifies kraft pulps has come under increasing scientific investigation (Bourbonnais et al., 1997a,b; Sealey and Ragauskas 1997; Poppius-Levlin et al., 1997; Potthast et al., 1997). In this report we present our findings on the structural activity correlation between delignification and structural modification of N-hydroxybenzotriazole.

Experimental

Materials and Methods

All reagent and specialty chemicals including N-hydroxybenzotriazole, benzotriazole, N-hydroxymethylbenzotriazole, 6-trifluoro-methylhydroxy-benzotriazole, N-hydroxypthalimide, N-hydroxymethylpthalimide, 3-hydroxy-1,2,3-benzotriazin-4(3H)-one and 2-hydroxy-benzimidazole were commercially obtained and used as received. An industrial southern U.S.A. softwood kraft pulp was employed for all biobleaching studies; prior to use, the pulp was extensively washed until the effluent was pH neutral and colorless. The kappa number of the washed pulp was 26.8 and its viscosity was 31.9 cP. 1-Hydroxy-4-methoxy-benzotriazole and 1-hydroxy-4-methylbenzotriazole were prepared following the procedure of Konig and Geiger (1970). Laccase, isolated from a *Polyporus* fungus, was provided by Novo Nordisk. The enzyme was frozen to -20°C until use. Once thawed, the activity of the enzyme was measured, and the proper dose was added to the pulp.

Laccase activity measurements

The activity of the laccase was measured by monitoring the rate of oxidation of syringaldazine by UV/Vis. The change in A_{530nm} of 0.001 per minute per mL of enzyme solution in 100 mM potassium phosphate buffer (2.2 mL) and 0.216 mM

syringaldazine in methanol (0.3 mL) was set to one unit (U) of activity. This test was performed at 23°C.

LMS delignification

A 1 litre pressure reactor containing 10% consistency pulp (10 g, o.d.) was warmed to 45°C. The mediator (1.48×10^{-3} mol) was added to the pressure reactor, and the mixture was stirred for 3 minutes. The pH was then adjusted to 4.5 with glacial acetic acid and the laccase solution (3.00 mL, $1{,}752 \times 10^{3}$ U) was added to this mixture. The pressure vessel was then sealed, pressurized with oxygen (145 psi), and then stirred for 24 h. The pulp was then removed from the reactor, washed with water, and alkaline extracted with 2% NaOH, at 70°C, for 1 hour in a sealed bag at 10% consistency The extracted pulp was washed with water, air dried, and analyzed for kappa number. The kappa number measurement was performed in accordance with TAPPI Useful Method UM-246 (Tappi, 1991) and each measurement was performed in duplicate (standard deviations ± 2%).

Computational Modeling

Computational work on the mediator compounds was performed at the semi-empirical level, using the PM3 Hamiltonian operator and full geometry optimizations (Stewart, 1990). Open-shell structures were optimized using the Unrestricted Hartree-Fock method, followed by a single-point calculation with the

half-electron approximation. The former method converges more rapidly, while the latter provides energies that can be compared with closed-shell structures. All calculations were done using Sybyl under license from Tripos Associates, running on a Silicon Graphics Indigo workstation in the School of Forestry at Auburn University.

Results and Discussion

The bleaching effect of N-hydroxybenzotriazole and laccase (Potthast et al., 1997) has been suggested to occur due to the in-situ formation of a N-hydroxy radical, as shown in Figure 2.

To explore the structural requirements of this mediator for laccase catalyzed delignification of kraft pulps we elected to explore the use of structural analogues of N-hydroxybenzotriazole. The mediators studied in this report are shown in Figure 3.

For the most part, the mediators that were examined for a LMS stage were chosen so as to evaluate the importance of the N-hydroxy group and proposed stability of the N-hydroxy radical as it affects the delignification properties of the LMS process. The efficiency of the laccase-mediator delignification process was explored by treating a southern softwood kraft pulp with standardized LMS biobleaching conditions for 24 h. Typically, a pulp slurry (10% csc) was preheated to 45°C, the mediator was added and the mixture was pH adjusted to 4.5 using glacial acetic acid. This mixture was vigorously stirred for 3 min prior to the addition of the enzyme. The pulp slurry was then sealed, pressurized to 10 barr O_2, and stirred for 24 hr at 45°C. After treatment the pulp was filtered, washed with water, and the fibers were extracted with an aqueous 0.5 N NaOH solution for 1 h at 70°C. The alkaline-extracted pulp was filtered, washed with water, air-dried, and its physical properties were determined.

Preliminary experiments summarized in Table 1 indicated that if either the mediator or the laccase was omitted from the LMS-stage, minor amounts of lignin were removed from the pulp presumably due to alkaline leaching. Nonetheless, examination of the extent of delignification when both laccase and N-hydroxybenzotriazole are present confirmed previous claims to this biobleaching system (Call and Mucke, 1996).

Interestingly, the use of compounds 2 or 3 as a mediator for laccase failed to yield any significant delignification of the kraft pulp. Clearly, the failure of 2 to act as an effective mediator for delignification supports the proposed mechanism of delignification involving a N-hydroxy radical. Presumably the insertion of a methylene group between the nitrogen and the hydroxy group raises the energy of activation for formation of the N-hydroxy radical too high so as to allow for its formation by laccase. The necessity of the N-hydroxy group is further validated by the observation that 1-H benzotriazole (compound 3) is also inactive as a mediator for laccase. Recently, Sealey and Ragauskas (1997) and others (Potthast et al., 1997, (Bourbonnais et al., 1997a) have reported that N-hydroxybenzotriazole is converted into 1-H benzotriazole during a LMS-stage and clearly this conversion is detrimental towards the overall delignification effect.

Biobleaching experiments also demonstrated that 3-hydroxy-1,2,3-benzotriazin-4(3H)-one or 2-hydroxybenzimidazole were ineffective as mediators for delignification of kraft pulps with laccase. Although several factors may be contributing to the lack of delignification with these two mediator structures, it appears that the laccase/N-hydroxybenzotriazole biobleaching system has rather high structural specificities for delignification to occur.

To explore the influence of the aromatic ring on the delignification properties of N-hydroxybenzotriazole, we examined the use of triazole derivatives 6 to 8. The introduction of a strong electron withdrawing group on the aromatic ring, such as trifluoromethyl, was detrimental with respect to the delignification process. Compound 7, with a 5-methyl group, was an active mediator for the delignification of kraft pulps but not as effective as N-hydroxybenzotriazole. Interestingly, the use of additive 8, with a 5-O-methoxy group, raised the apparent kappa number of the pulp after the LMS-stage. This was tentatively attributed to the generation of a reactive intermediate that coupled to the kraft pulp thereby affording a higher kappa number than the initial value.

Given the rather stringent limitations placed on the N-hydroxy benzotriazole structure for effective delignification to occur during a LMS stage we explored several alternative N-hydroxy mediator structures. One of our first candidates to be examined was N-hydroxyphthalimide. Literature results by Ishii, et al. (1996) have shown that this reagent can be employed as a co-oxidant in the presence of Co(acac) and oxygen. A variety of structures have been oxidized by this system including benzylic alcohols, and primary and secondary alcohols. The active oxidizing agent in this system is believed to be the N-hydroxy radical of N-hydroxyphthalimide. Given the clear similarities with the proposed mechanism for delignification by laccase and N-hydroxybenzotriazole, it was of interest to determine if N-hydroxyphthalimide could act as a mediator for laccase.

Employing the same pulp and experimental conditions as described above, the kraft pulp was treated with N-hydroxyphthalimide and laccase for 24 hr. Following the usual alkaline extraction procedure, we determined that the pulp had experienced a 28% decrease in kappa number. Although this is not as substantial a decrease in kappa number as observed with N-hydroxybenzotriazole, it is certainly much more than was observed with any other benzotriazole derivative. Interestingly, insertion of a methylene group between the nitrogen and the hydroxy group of N-hydroxyphthalimide halted the delignification capabilities of this mediator (see Table 1, compound 10). These results support the suggestion that effective delignification with N-hydroxy mediators requires a covalent bond between these two functional groups.

To determine if a simpler pyrrolidine or maleimide structure could act as an effective mediator for laccase, we examined the biobleaching chemistry of additives 11 and 12 (see Table 1). In each case, we were unable to measure any significant delignification in the laccase mediator bleaching treatment. Presumably, the N-hydroxy radical generated in this process needs the additional delocalization capabilities available in N-hydroxyphthalimide to become an effective delignification agent.

Computational Modeling

The calculated bond dissociation energies for the dehydrogenation of mediator

compounds (Figure 2) are as shown in Table 2. This is, of course, a thermodynamic term describing the energy required for the generation of the free radicals. The additional values reported in Table 2, the energy of the singly occupied molecular orbital (SOMO), charge, spin density and SOMO density, all refer to the mediator radicals and are measures of reactivity toward the substrate.

Given the intrinsically soft nature of radicals (Fleming 1976), it is proposed that their reactions are under frontier orbital control, in this case relying on the interaction of the SOMO of the radical with the HOMO (highest occupied molecular orbital) of the substrate. If creosol is considered as a lignin model, with a HOMO energy of –8.783 eV, it can be seen that all of the SOMO energies of the mediators are lower, such that the higher values of the latter will minimize the HOMO-SOMO gap, promoting the reaction in question. Furthermore, in frontier molecular orbital theory, the magnitude of the orbital coefficients for the reactive center, as measured by the SOMO density, is reported as an important factor in determining relative reactivity. More familiarly, total charge may be taken as a measure of nucleophilicity and the spin density is an assessment of amount of unpaired spin character at the site of reaction. For these radical species, charge, SOMO density, and spin density were measured at the atom from which the hydrogen was removed (i.e., the nitrogen in compound 3 and oxygen in all other compounds).

Among compounds 1-5, it was found experimentally that only N-hydroxybenzotriazole was an effective delignification agent. In accord with this result, the bond dissociation energy for compound 1 is the lowest among these compounds, the SOMO-HOMO energy gap is the smallest, the SOMO density is largest, and it has the largest partial negative charge. Interestingly, the unpaired spin density of compound 1 is the lowest among compounds 1-5. This may be indicative of increased delocalization of the single electron that is less likely among the other compounds.

The SOMO energies of compounds 6-8, relative to N-hydroxybenzotriazole, are in concert with the predictions of frontier molecular orbital theory, in which electron withdrawing groups and electron donating groups will lower and raise this energy term, respectively. Compound 7 was found to be an active compound and indeed its bond dissociation energy is only slightly below (1), while both the SOMO energy and partial negative charge are both slightly larger than those for compound 1. The observed behavior of compound 8 is somewhat puzzling since, based on the theoretical results, it is comparable and, in some cases, better than N-hydroxybenzotriazole. Perhaps these data are consistent with the proposed formation of reactive intermediates that might couple with the lignin to give an elevated kappa number.

Within the phthalimide compounds, the most efficacious with respect to delignification is compound 9, which among its analogues (9-12) has the lowest bond dissociation energy, largest partial negative charge. Indeed the values reported for compound 9 are not markedly different from N-hydroxybenzotriazole itself. Compound 9 also has the lowest spin density which again may be interpreted in terms of increased delocalization as proposed earlier.

Given the undoubted complexity of the reactions between the mediators and lignin, it might have been expected that the overall reactivity is controlled by a combination of thermodynamic and kinetic factors. Based on the bond dissociation energies and other electronic reaction indices it appears that this is the case. In general, however, among the mediators examined and factors evaluated, N-hydroxybenzotriazole would have been predicted to be an effective delignification agent.

Conclusions

The studies presented in this paper highlight the rather stringent requirements for delignification of kraft pulps with laccase and N-hydroxybenzotriazole like structures. The results of these investigations support the proposed importance of N-hydroxy radicals as the active delignification agent for kraft pulps. The

observation that both compounds 7 and 10 were mediators for laccase (albeit less effective that N-hydroxybenzotriazole) suggest that the steric requirements about the active site of laccase are not overly specific, which is also consistent with the ability of the enzyme to oxidize ABTS. Recently, Amann (1997) has reported that 1-hydroxy-1,3,5-triazaperhydroine-2,4,6-trione and N-hydroxy-N-phenylacetamide were mediators for laccase with improved delignification properties for chemical pulps. Results from both the experiment and the calculation support the suggestion that the steric requirements about the active site of laccase are not stringent; but that other factors, such as the electronic nature of the oxygen radical, contribute to the observed delignification properties. In additional to these considerations the delignification capabilities of a LMS-stage are also influenced by the overall stability of the mediator and the enzyme as suggested by Bourbonnais et al., (1997a,b) and Sealey and Ragauskas (1997).

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Table 1. Biobleaching results employing a laccase mediator system for delignifying a 26.8 kappa number softwood kraft pulp[1].

Mediator	**% Delignification**	**Mediator**	**% Delignification**
No Mediator	14	6	10
1 + no Laccase	9	7	23
1	41	8	-15
2	12	9	28
3	8	10	11
4	12	11	16
5	17	12	10

[1]% delignification values were determined by measuring the initial kappa number and the value after LMS.

Table 2. Results from computational modeling of potential laccase mediators.

Compound	Bond dissociation energy (kcal/mole)	SOMO energy (eV)	Charge	Spin density	SOMO density
1	79.066	-9.2831	-0.405	0.455	0.234
2	104.966	-9.3252	-0.182	0.876	0.007
3	97.112	-10.2602	-0.020	0.719	0.195
4	80.842	-9.3055	-0.269	0.601	0.057
5	85.962	-9.7048	-0.347	0.803	0.040
6	80.224	-9.6665	-0.391	0.447	0.238
7	79.219	-9.1807	-0.409	0.430	0.120
8	78.825	-9.0829	-0.413	0.430	0.180
9	80.315	-9.7392	-0.365	0.533	0.341
10	97.625	-10.3673	-0.190	0.878	0.018
11	81.011	-9.9004	-0.357	0.541	0.339
12	80.514	-9.9453	-0.358	0.536	0.348

[1]all calculated bond dissociation energies and other radical properties are based on the RR'N-O• species with the exception of proposed mediator 3 for which the hydrogen of N-1 was removed.

Fig. 1. Comparative study of laccase (400 x 10^3 units of activity) catalyzed delignification of a post-oxygen delignified softwood kraft pulp (kappa number: 17.2) employing no mediator, ABTS, and N-hydroxybenzotriazole (1.50 x 10^{-3} mols mediator). Percentage delignification determined after laccase treatment and alkaline extraction stage (Sealey and Ragauskas, 1998).

Fig. 2. Proposed active delignification agent for laccase/N-hydroxybenzotriazole bleaching system (Potthast, Koch, and Fischer, 1997).

Fig. 3. Mediators employed for LMS-stage.

Figure 1

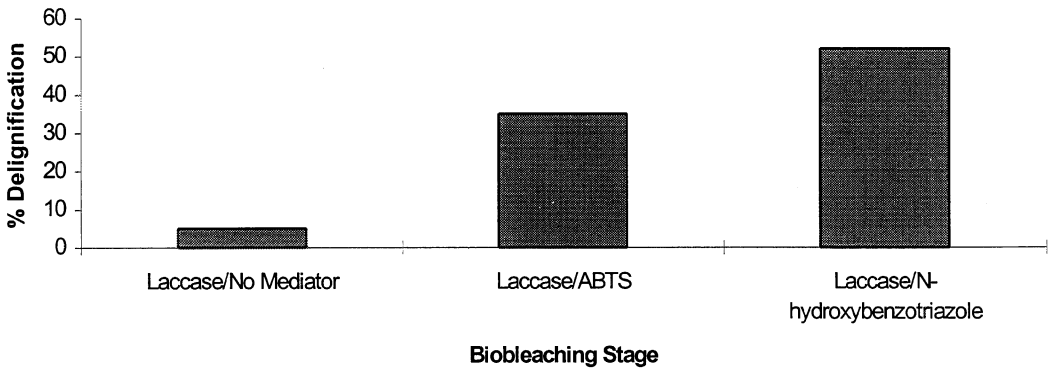

60
50
40
30
20
10
0
% Delignification
Laccase/No Mediator
Laccase/ABTS
Laccase/N-hydroxybenzotriazole
Biobleaching Stage

Figure 2

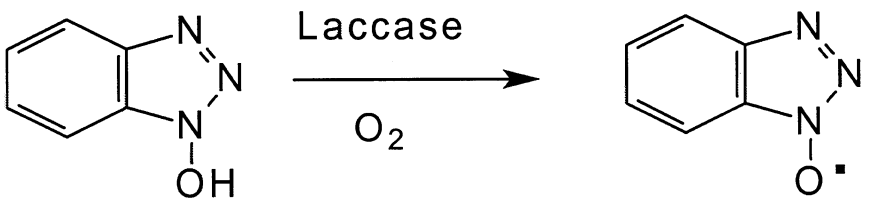
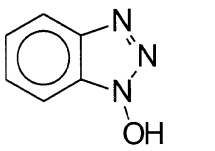


Figure 3



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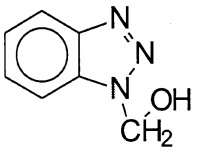

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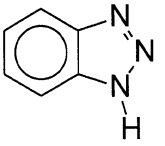

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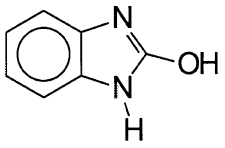

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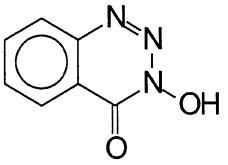

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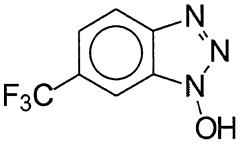

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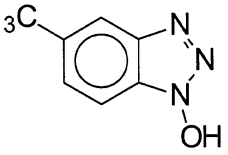

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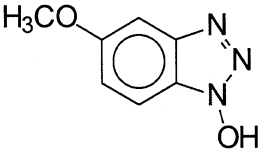

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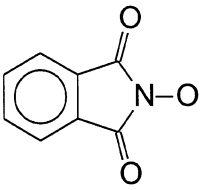

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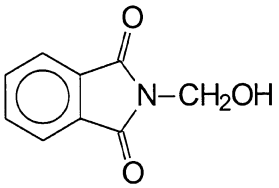

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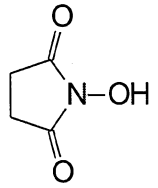

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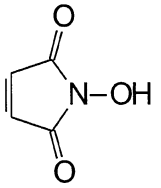

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